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Review

Adenosine receptors and cancer ☆

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ABSTRACT

Adenosine is a ubiquitous signaling molecule whose physiological functions are mediated by its interaction with four G-protein-coupled receptor subtypes, termed A_1, A_{2A}, A_{2B} and A_3. As a result of increased metabolic rates, this nucleoside is released from a variety of cells throughout the body in concentrations that can have a profound impact on vasculature and immunoescape. However, as high concentrations of adenosine have been reported in cancer tissues, it also appears to be implicated in the growth of tumors. Thus, full characterisation of the role of adenosine in tumor development, by addressing the question of whether adenosine receptors are present in cancer tissues, and, if so, which receptor subtype mediates its effects in cancer growth, is a vital research goal. To this end, this review focuses on the most relevant aspects of adenosine receptor subtype activation in tumors reported so far. Although all adenosine receptors now have an increasing number of recognised biological roles in tumors, it seems that the A_{2A} and A_3 subtypes are the most promising as regards drug development. In particular, activation of A_{2A} receptors leads to immunosuppressive effects, which decreases anti-tumoral immunity and thereby encourages tumor growth. Due to this behavior, the addition of A_{2A} antagonists to cancer immunotherapeutic protocols has been suggested as a way of enhancing tumor immunotherapy. Interestingly, the safety of such compounds has already been demonstrated in trials employing A_{2A} antagonists in the treatment of Parkinson's disease. As for A_3 receptors, the effectiveness of their agonists in several animal tumor models has led to the introduction of these molecules into a programme of pre-clinical and clinical trials. Paradoxically, A_3 receptor antagonists also appear to be promising candidates in human cancer treatment of regimes. Clearly, research in this still field is still in its infancy, with several important and challenging issues remaining to be addressed, although purine scientists do seem to be getting closer to their goal: the incorporation of adenosine ligands into drugs with the ability to save lives and improve human health. This article is part of a Special Issue entitled: "Adenosine Receptors".
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Contents

1. Introduction

The purine nucleoside adenosine is consensually identified as a major local regulator of tissue function especially when energy supply fails to meet cellular energy demand. Due to its ability to equalize energy intake to metabolic demand, in the 1980s it earned the reputation of a

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"retaliatory metabolite" [1]. Adenosine is omnipresent, released from almost all cells, and generated in the extracellular space by breakdown of ATP through a series of ectoenzymes, including apyrase (CD39) and 5′-nucleotidase (CD73) [2]. The latter dephosphorylates extracellular AMP to adenosine, regulating the limiting step for its formation. Extracellularly, adenosine concentration is kept in equilibrium by reuptake mechanisms operated through the action of specific transporters. Then inside the cell it is phosphorylated to AMP by adenosine kinase or degraded to inosine by adenosine deaminase (ADA). Intracellularly, adenosine formation is dependent upon the hydrolysis of AMP by an intracellular 5-nucleotidase or hydrolysis of *S*-adenosylhomocysteine. It is estimated that the levels of adenosine in the interstitial fluid are in the range 20–200 nM [3]. Adenosine concentrations increase under metabolically unfavorable conditions. Tissue hypoxia, for example, leads to an enhanced breakdown of ATP and an increased generation of adenosine. In addition to this route, the release of adenosine might be potentiated by hypoxia-dependent inhibition of the salvage enzyme adenosine kinase which rephosphorylates the nucleoside to AMP [4]. As adenosine is unstable and its half-life is limited by deamination or cellular reuptake, hypoxia-induced increase typically affects only local adenosine receptor (AR) signaling. Adenosine effects are widespread and pleiotropic. The cellular response to this autacoid strictly depends on the expression of the different AR subtypes, which can be co-expressed by the same cell and serve as active modulators in signal transduction. ARs have been actively studied as potential therapeutic targets in several disorders such as Parkinson's disease, schizophrenia, analgesia, ischemia, and cancer. Indeed, an overview of the recent advances reported regarding the implication of ARs in the latter disease is the focus of this review.

2. Adenosine receptors

Adenosine mediates its effects through activation of a family of four G-protein-coupled ARs, named A_1, A_{2A}, A_{2B}, and A_3. These receptors differ in their affinity for adenosine, in the type of G proteins that they recruit, and finally in the downstream signaling pathways that are activated in the target cells. A_1, A_{2A} and A_3 ARs display high affinity while A_{2B} displays low affinity for adenosine [5,6]. These receptors are seven transmembrane glycoproteins coupled with G proteins and are widely distributed throughout the body. A_1AR stimulation through interaction with different members of pertussis toxin-sensitive family of G proteins (Gi1, Gi2, Gi3 and G0) may modulate adenylyl cyclase (AC), calcium channels, potassium channels and phospholipase C (PLC). The A_{2A}AR through coupling with Gs/Golf proteins activates AC activity and its stimulation increases the intracellular cyclic adenosine monophosphate (cAMP) concentration. The A_{2B}AR, through coupling with Gs/Gq protein, increases activity of AC and stimulates PLC. Finally, the A_3AR through interaction with Gi and Gq proteins inhibits AC and stimulates PLC, respectively [7]. Furthermore, depending on the cell type studied, ARs may also signal through the activation of mitogen-activated protein kinase (MAPK) signaling pathway. The MAPK pathways are critically important in the regulation of cell proliferation and differentiation [8]. There are numerous extracellular signals feeding into these cascades, including input via GPCRs [9]. All four subtypes of ARs have been shown to mediate extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in transfected Chinese hamster ovary (CHO) cells [10,11]. MAPK signaling and hence cell proliferation might be amenable to manipulation through specific ARs in tumor cells. Recently, different classes of proteins have been recognized as interacting with ARs. These proteins include serine – threonine or tyrosine protein kinases, β-arrestins and scaffolding proteins within others [5].

3. Adenosine receptors (ARs) and cancer

One of the first evidence concerning the involvement of adenosine in cancer comes from the studies in immune cells. It is known that the ability of immune cells to fight tumor cells is fundamental for successful host defence against cancer. Adenosine, whose concentration increases within hypoxic regions of solid tumors, has been recognized able to interfere with the recognition of tumor cells by cytolytic effector cells of the immune system [12,13]. Adoptive immunotherapy with lymphokine-activated killer (LAK) cells has shown some promise in the treatment of certain cancers that are unresponsive to conventional treatment approaches. However, colon adenocarcinomas tend to respond poorly to LAK therapy, possibly as a result of tumor-induced immunosuppression. It has been demonstrated that colon adenocarcinoma cells inhibited anti-CD3-activated killer cell induction through the production of a tumor-associated soluble factor that was distinct from transforming growth factor-beta (TGF-β) or prostaglandins [14]. Therefore, adenosine was indicated as a possible inhibitor of killer T-cell activation in the microenvironment of solid tumors [15,16]. Indeed, evaluating the adhesion of murine spleen-derived anti-CD3-activated killer (AK) lymphocytes to syngeneic MCA-38 colon adenocarcinoma cells, it was found that adenosine reduced adhesion by up to 60% [17]. The inhibitory effect of adenosine was exerted on AK cells and not on the MCA-38 targets and the agonist potency profile indicated that the A_3AR subtype might be responsible for the inhibition of adhesion. It has been suggested that this mechanism of immunosuppression, secondary to tissue hypoxia, may be important in the resistance of colorectal and other solid cancers to immunotherapy. In addition it has been demonstrated that adenosine plays a strong inhibitory role on the induction of mouse cytotoxic T-cells [18]. The inhibitory effect of adenosine on AK-T cell proliferation was also blocked by an A_3AR antagonist suggesting that adenosine stimulates A_3ARs to prevent AK-T cell induction. Tumor-associated adenosine may act through the same mechanism to impair the development of tumor-reactive T cells in cancer patients. Therefore the suppression of T-killer cell function suggests that adenosine may behave as a local immunosuppressant within the microenvironment of solid tumors. Subsequently, it was reported that adenosine partially inhibits the interaction of T lymphocytes with tumor cells by blocking the function of integrin α4β7 that is the major cell adhesion molecule involved in the adhesion of T cells to syngeneic MCA-38 adenocarcinoma cells [19]. The effect of adenosine on the expression of costimulatory molecules by T cells in resting and activated conditions has been investigated. One of the most important costimulatory molecules present on the T cells' surface are CD2 and CD28 acting in concert to achieve optimal costimulation of T lymphocytes during interaction with antigen presenting cells. It has been demonstrated that adenosine interfered with activation-induced expression of the costimulatory molecules CD2 and CD28 in a way IL-2 dependent, but not involving the accumulation of intracellular cAMP, possibly by activating the A_3 subtype [20]. However, other data obtained from studies using AR knock-out (KO) mice examined the capability of adenosine and its analogues to inhibit the ability of LAK cells to defeat tumor cells. This work demonstrated that adenosine and adenosine A_{2A} ligands suppress the cytotoxicity of LAK cells in parallel with their ability to increase cAMP levels. Studies with LAK cells generated from A_1 and A_3AR KO mice indicated the lack of any involvement of these adenosine subtypes in the inhibitory effect exerted by adenosine, whereas LAK cells obtained from A_2AR KO mice were resistant to the inhibitory effect of the nucleoside. Only very high concentrations of the non-selective agonists 5-N-ethylcarboxamide adenosine (NECA) or 2-chloroadenosine (CADO) produced mild inhibition of LAK cytotoxicity that were possibly induced through A_{2B} activation, suggesting the predominant role of the A_{2A} subtype in inhibition of LAK cell toxicity [21]. Therefore, the use of A_{2A} antagonists has been suggested to increase the efficacy of immunotherapy [22].

In order to shed light on the role of adenosine in cancer, we report here the plethora of data performed in "in vitro" and in "in vivo" studies for each AR subtype.

4. Role of A_1 adenosine receptors (A_1ARs) in cancer

In spite of the various studies investigating the role of A_1AR in tumor development its role is not well characterized. A_1ARs have been associated to carcinogenesis in previous investigations where the expression of this receptor has been demonstrated in colorectal adenocarcinomas and peritoneal colon tissues [23]. A_1ARs have been detected also in the human leukemia Jurkat and human melanoma A375 cell lines [24,25]. Despite this association, in human LoVo metastatic cell line A_1 agonists inhibited proliferation [26] and similar antiproliferative effects were reported for TM4, a Sertoli-like cell line [27]. Proliferation in human tumor cells (leukemia MOLT-4, breast tumors T47D, HS578T and MCF-7) was inhibited by A_1 stimulation. On the other hand, the A_1AR, endogenously expressed in melanoma cell line, has been demonstrated to increase the chemotaxis of tumor cells. Otherwise, unresponsive CHO cells transfected with the adenosine A_1AR cDNA (CHO-hA_1) acquired the direct, pertussis toxin-sensitive, chemotactic response to adenosine, and this response was inhibited by AR antagonists [28]. These findings demonstrate that adenosine and adenine nucleotides are capable of stimulating chemotaxis of tumor cells probably through the A_1AR subtype. Consistent with a protumoral effect of the A_1AR, it has been reported that human primary breast tumor tissues express higher levels of A_1ARs than in matched normal breast tissues. A_1ARs have been detected also in MDA-MB-468 human tumor cell lines where depletion of A_1ARs attenuates both cell growth and cell proliferation. Cell cycle analysis indicated that depletion of A_1ARs by siRNA impairs G1 checkpoint, leading to marked accumulation of cells in G2/M phase, in agreement with the inhibitory effect on cell proliferation. Further supporting this finding, synchronization studies of HeLa cells in various cell cycle phases suggest that A_1AR expression is suppressed in G2/M cells and depletion of A_1AR expression by siRNA produced differential expression of several key cell cycle regulators, i.e., accumulation of the cyclin-dependent kinase inhibitor p27 with concomitant reduction of CDK4 and cyclin E proteins. In addition to the impact on cell cycle progression, depletion of A_1ARs by siRNA results in substantial cell death and apoptosis. Together these findings suggest that the A_1AR may contribute to tumor cell growth and survival in breast tumor cells [29].

The density of A_1ARs in F98 glioma-bearing rats has been found to be increased in zones surrounding experimental tumors. Immunostaining identified activated astrocytes as the main origin of peritumoral A_1 upregulation. These results have been confirmed in a pilot study with 8-cyclopentyl-3-(3-^{18}F-fluoropropyl)-1-propylxanthine (^{18}F-CPFPX) using PET on a patient with recurrent glioblastoma multiforme [30]. The distribution of A_1 in rat brain tumors and in rat C6 glioma cells has been confirmed in different studies [31,32]. In particular, it has been reported that adenosine-mediated inhibition of glioblastoma growth is dependent on activation of A_1 in microglial cells [33]. This implies that adenosine, acting via A_1AR, impairs glioblastoma growth.

Accordingly, it has been reported that in rat astrocytoma cells extracellular adenosine appears to activate caspase-9 followed by the effector caspase-3, at least via two independent pathways linked to A_1AR-mediated AC inhibition and adenosine uptake into cells. This study represents a new pathway for caspase activation relevant to diverse adenosine signals in cell death [34].

More recently, it has been shown that extracellular adenosine-induced apoptosis of CW2 human colonic cancer cells by activating caspase −3, −8 and −9, and the effect was inhibited by an A_1AR antagonist. For mice inoculated with CW2 cells, intraperitoneal injection with adenosine reduced tumor growth by inducing apoptosis mediated via A_1ARs [35]. The results from the studies described above suggest that the A_1AR plays an antitumorigenic role in the development of glioblastomas. Further research into the mechanisms of how the pathways of A_1AR signaling modulate glioblastoma development may ultimately lead to treatments to reduce the progression of this disease.

The pro- and anti-tumoral effects induced by A_1AR activation are summarized in Fig. 1.

5. Role of A_{2A} adenosine receptors (A_{2A}ARs) in cancer

A_{2A}ARs have been found on cell membranes of different human tumor cells: SH-SY5Y neuroblastoma, NG108-15 neuroblastoma x glioma hybrid, U937 monocytic lymphoma, Jurkat T-cell leukemia, A375 melanoma, A431 epidermoid cells, colon carcinoma HT29 and DLD-1 cells, U87MG human glioblastoma cells, and human breast cancer MCF-7 cells [24,25,36–43].

Treatment with an adenosine A_{2A} agonist results in a reduction in neuronal apoptosis and a decrease in spinal cord reperfusion inflammatory stress during rabbit spinal cord reperfusion [44]. It is possible to achieve infarct reduction with adenosine, inhibiting apoptosis. The inhibition of apoptosis by adenosine at reperfusion involves the

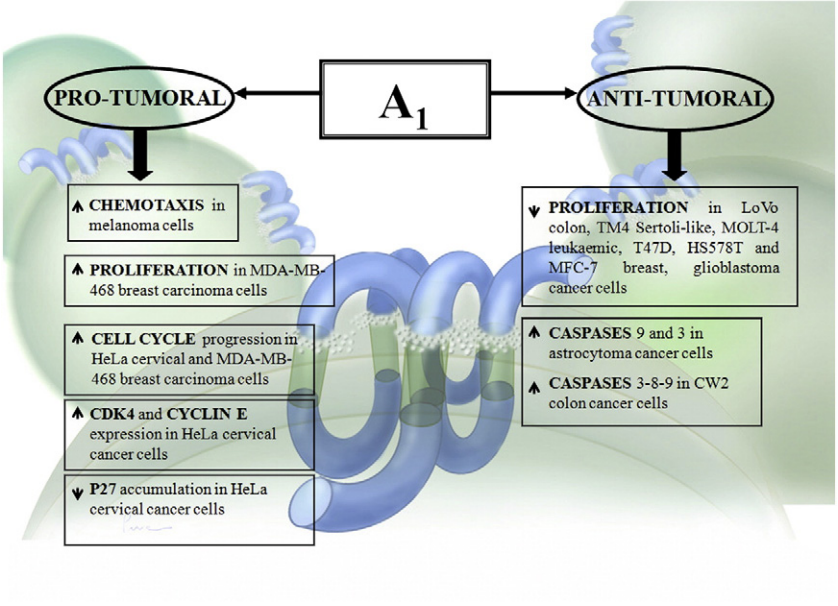

Fig. 1. Role of A_1AR in cancer development. Schematic representation of the principal pathways activated by A_1AR in the modulation of pro- and anti-tumoral effects.

alterations in anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins and neutrophil accumulation, primarily mediated by an A_{2A}AR [45].

Despite this anti-apoptotic role on normal tissue, activation of adenosine A_{2A}AR has been shown to promote cell death of human A375 melanoma cells [46]. Accordingly, it has been reported that extracellular adenosine induces apoptosis by activating caspase-9 and caspase-3 in association with mitochondrial damage via A_{2A}ARs in Caco-2 human colonic cancer cells [47].

Furthermore, adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A_{2A}ARs [48]. Angiogenesis is the requisite not only for continued tumor growth, but also for metastasis. Without access to the vasculature, the tumor cells cannot metastasize. Hence, angiogenesis is a necessary biologic feature of malignancy. Several studies have revealed a correlation between the extent of angiogenesis (microvessel density) and the probability of metastases in melanomas and cancers of the breast, lung, colon, and prostate. In some of these cancers (breast, prostate) vessel density has proven to be a significant prognostic indicator. A_{2A}AR occupancy promotes endothelial cell proliferation, migration and synthesis of message for the important angiogenic growth factor such as vascular endothelial growth factor (VEGF) [48–51]. These data suggest that A_{2A}ARs are involved in promotion of angiogenesis. Thus, it has been hypothesized that A_{2A}AR antagonists may exert anti-tumoral effects by impairing solid tumor growth and spreading.

On the other hand, in the vasculature, A_{2A}ARs have been detected on both the smooth muscle and endothelium, where they are associated with vasodilatation [52]. Agonists of A_{2A}ARs may be used to improve vasodilatation of intratumoral blood vessels enhancing delivery of anticancer drugs into the tumor tissue. This may be particularly important for malignant brain tumors where the limited effectiveness of chemotherapy has been related to insufficient drug delivery into the tumor parenchyma. The results presented above provide further evidence for an active role of A_{2A}ARs in tumor growth.

A_{2A}ARs have been shown to increase erythropoietin (EPO) production in hepatocellular carcinoma (Hep3B) cells in culture and in vivo in rats and mice under normoxic and hypoxic conditions [53,54]. It has been reported that renal cell carcinoma, hepatocellular carcinoma and cerebellar hemangioblastoma are EPO-secreting tumors. This inappropriate (pathologic) secretion of EPO may cause secondary polycythenias, an increase in red cell mass. It has been demonstrated that adenosine activates EPO production through the A_{2A}ARs, suggesting that the adenosinergic system may allow gaining a deeper insight into the pathophysiology of this disorder. In particular, A_{2A} antagonists may be a reasonable therapeutic target for stopping increased levels of EPO.

It is well known that adenosine is involved in the inhibition of immune function [55,56]. In particular A_{2A}ARs on T-cell surface may play an immunosuppressive role in large solid tumors where hypoxic conditions are known to cause accumulation of extracellular adenosine, which, in turn, could inhibit incoming antitumor cytotoxic T lymphocytes from destroying the tumor [57]. The immunosuppressive role and the ability to protect against ischemia suggest that A_{2A}AR activation improves hypoxic tumor cell survival and immuno escaping. A role for the A_{2A}AR in protecting host tissue from destruction by overexuberant immune responses has been established. Considering that the tumor microenvironment contains relatively high levels of extracellular adenosine, data support the hypothesis that tumor-derived adenosine is one mechanism by which tumors evade immune destruction [12,15,58]. In particular, the important work by Ohta and Sitkovsky [59] established for the first time the nonredundant role of the A_{2A}AR in mediating adenosine-induced anti-inflammatory responses in A_{2A}AR KO mice [59]. These KO mice are unable to control inflammation, which leads to extensive, often fatal, tissue destruction. For example, when immune-mediated liver inflammation was induced in mice by injecting Con A, the A_{2A}AR KO mice died because of massive immune-mediated liver destruction. These observations led to a model whereby tissue damage resulting from inflammation leads to the release of extracellular adenosine, which then acts to reduce the inflammatory response by acting on bone marrow-derived immune cells. Indeed, A_{2A}AR signaling on immune cells such as macrophages, T cells and dendritic cells has been shown to limit effector cell function [60–66]. The existence of this negative feedback loop has led Sitkovsky to propose that, from an immunologic prospective, adenosine should be viewed as a metabokine that acts as an inhibitory second signal [67,68].

Furthermore adenosine acting via the A_{2A}AR has the ability to influence inflammation by inhibiting proinflammatory cytokine secretion, C2 activation, macrophage-mediated phagocytosis, and superoxide production [69]. Likewise, A_{2A}AR activation has profound effects on the adaptive immune response. A_{2A}AR activation inhibits both CD4+ and CD8+ T-cell function [60,63,64,70,71]. Interestingly, A_{2A}AR activation on T cells seems to selectively inhibit proinflammatory cytokine expression while sparing anti-inflammatory cytokine expression [64].

Along these lines, it has been shown that adenosine acting via the A_{2A}AR might partially mediate the suppressive function of regulatory T (Treg) cells, crucial for maintaining tolerance to self and thus preventing autoimmune diseases and allograft rejections, by engaging the A_{2A}ARs on the suppressed cells [72]. It was found that the ectoenzymes CD39 and CD73 appear to be more specific markers for Treg cells than CD25 [72]. In particular, TCR-activated Treg cells express A_{2A}AR, A_{2B}AR and hypoxia-inducible factor (HIF-1) which leads to the enhanced transcription of genes that have hypoxia and/or cAMP-response elements (HRE and CRE, respectively). These genes, e.g. IL-10, TGF-β, Galectin-1, and Foxp3 have been implicated in Treg cell function and development [68]. Therefore, A_{2A}R stimulation not only inhibits the generation of adaptive effector T cells but also promotes the induction of adaptive Treg cells. A_{2A}AR signaling during an initial antigen encounter can induce a state of anergy in primary T cells such that they are hyporesponsive on rechallenge. T cells initially stimulated in the presence of an A_{2A}R agonist fail to proliferate and produce IL-2 and interferon (IFN)-γ when rechallenged in the absence of A_{2A}R stimulation. Likewise, in an in vivo model of autoimmunity, tissue-derived adenosine promotes anergy and abrogates tissue destruction. Indeed, A_{2A}R stimulation inhibits IL-6 expression while enhancing the production of TGF-β. Accordingly, treating mice with A_{2A}R agonists not only inhibits Th1 and Th17 effector cell generation but also promotes the generation of Treg cells [71]. The generation and accumulation of immunosuppressive adenosine by human Treg cells has been confirmed in recent studies [73,74]. Importantly, A_{2A}AR KO mice exhibit enhanced antitumor immune responses by $CD8^+$ T cells, as well as a dramatic reduction in the growth of experimental tumors in comparison to wild-type controls. A_{2A}AR signaling has also been implicated in adenosine-mediated inhibition of cytokine production and cytotoxic activity by activated natural killer (NK) cells [75].

Promising strategies to enhance immunosurveillance of cancer will come by the development of A_{2A}AR antagonists that may interfere with the ability of extracellular adenosine to induce a local immunosuppression in tumors. Indeed, it has been recently reported that adenosine-resistant effector T cells were successfully obtained by exposure of activated T cells to NECA. These in vitro studies form the basis for future attempts to produce antitumor T cells that are more effective in adoptive immunotherapy [76].

Finally, the A_{2A} agonist stimulates the proliferation of MCF-7 cells and interferes with the ethanol-induced activation of estrogen receptor (ER) signal transduction, opening a new perspective for the therapy of estrogen-dependent breast cancer [43].

The pro- and anti-tumoral effects induced by A_{2A}AR activation are summarized in Fig. 2.

6. Role of A_{2B} adenosine receptors (A_{2B}ARs) in cancer

The distribution of the gene transcript indicates that the A_{2B}AR is expressed in many tissues and cell types. However, it is clear that the

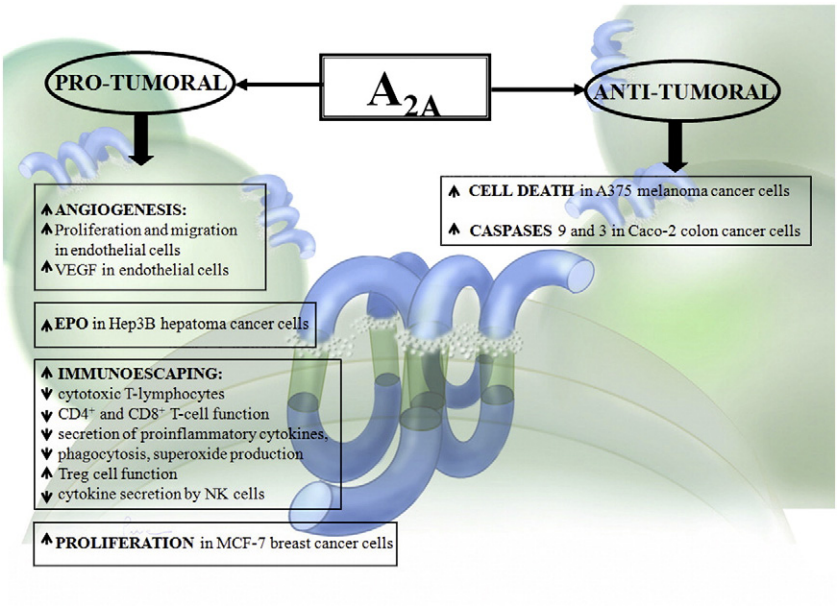

Fig. 2. Role of A_{2A}AR in cancer development. Schematic representation of the principal pathways activated by A_{2A}AR in the modulation of pro- and anti-tumoral effects.

receptor is activated only at high concentrations of adenosine, i.e., under pathophysiological rather than physiological conditions [3,5].

Extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A_{2B}AR, involving a cAMP dependent pathway [77]. Adenosine regulates macrophage proliferation by interacting with the A_{2B}AR and subsequently inducing the production of cAMP [78].

A_{2B}ARs are predominantly expressed in human microvascular cells and are believed to modulate expression of angiogenic factors [79]. In particular, adenosine activates the A_{2B}AR in human retinal endothelial cells (HRECs), which may lead to neovascularization by a mechanism involving increased angiogenic growth factor expression [80]. In regard to this, A_{2B}AR inhibition may offer a way to inhibit retinal angiogenesis and provide a novel therapeutic approach to the treatment of diseases associated with aberrant neovascularization, such as diabetic and prematurity retinopathy [81]. In light of this, antagonists for A_{2B}ARs appear to be useful in vivo to impair neovasculogenesis associated to tumor development and growth.

Adenosine causes growth inhibition of cardiac fibroblasts, aortic and vascular smooth muscle cells by the activation of A_{2B}ARs [82–86], while it increases the proliferation of rat arterial endothelial cells via A_{2B}ARs [87]. It is interesting to note that, in contrast to smooth muscle cells, A_{2B}ARs induce growth of endothelial cells [80]. In view of the fact that A_{2B}AR stimulation improves cell proliferation of the peripheral microvessels, we can hypothesize that, as a consequence of this ability to promote the endothelial cell proliferation, A_{2B} may contribute to tumor growth and spreading by inducing neovascularization in the area surrounding the tumor masses. The A_{2B}AR also seems to be responsible for the release of a certain subset of cytokines [88,89]. A_{2B}ARs are expressed in human microvascular endothelial cells, where they play a role in the regulation of the expression of angiogenic factors like VEGF, interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) [79]. Moreover, in human mast cells (HMC-1) derived from a highly malignant, undifferentiated human mastocytoma cancer, activation of A_{2B}ARs induces the release of IL-8 and VEGF, and the activation of A_3ARs induces angiopoietin 2 expression [88]. However, capillary formation induced by HMC-1 media was maximal when both HMC-1 A_{2B}ARs and A_3ARs were activated. Activation of A_{2B}ARs alone was less effective, suggesting cooperation between A_{2B}ARs and A_3ARs on HMC-1 cells to produce angiogenesis. Furthermore, in HT29 human colon cancer cells adenosine increases IL-8 expression via stimulation of A_{2B}ARs, while the stimulation of A_3AR caused an increase in VEGF [89]. In the glioblastoma cell line U87MG, a similar A_{2B}AR-mediated increase of IL-8 was observed [90]. In addition, it was shown that hypoxia caused an upregulation of A_{2B}ARs in these tumor cells. As these findings point to a crucial role for A_{2B}ARs in mediating the effects of adenosine on angiogenesis, blockade of A_{2B}ARs may limit tumor growth by limiting the oxygen supply.

Data demonstrating A_{2B}AR-mediated modulation of neovascularization may have interesting implications in the identification of novel drugs that, blocking A_{2B}ARs, may be utilized to induce protection against tumors.

A_{2B}ARs are also involved in the mRNA and protein increase of IL-6 in human astroglioma cells [91].

It has been reported that the ER-positive MCF-7 cells appeared to be devoid of any detectable amount of ARs, whereas the ER-negative MDA-MB-231 cells express very high levels of A_{2B}ARs. This AR subtype is responsible for the unusual inhibitory signal on ERK 1/2 phosphorylation. Moreover, antagonists like 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) block this response, confirming the identity of the AR subtype as the A_{2B}AR mediating the inhibition of ERK 1/2 phosphorylation [92]. The exact pathway leading to A_{2B}AR-mediated inhibition is not fully understood at this point. Both the Ca^{2+} signal detected following A_{2B}AR stimulation in MDA-MB-231 cells [92] and PLC activation are sufficient, as their blockade abolishes the inhibition of ERK 1/2 phosphorylation. A few studies describe such an uncommon antiproliferative GPCR-mediated signal in glomerular mesangial cells [93] and in vascular smooth muscle cells [85]. The high expression levels of A_{2B}ARs in an ER-negative breast cancer cell line together with a link to an antiproliferative signaling pathway make this AR subtype a potentially interesting target for tumor treatment, perhaps in combination with drugs interfering with downstream effectors in MAPK signaling pathways [94]. There is an increasing amount of data confirming that A_{2B}ARs play an important role in mediating the effects of adenosine on tumor growth and progression. The effects which are most interesting for a potential anticancer treatment based on A_{2B}ARs as a target are inhibition of angiogenesis and inhibition of ERK 1/2 phosphorylation. The dilemma is, however, that inhibition of angiogenesis requires the use of A_{2B}AR antagonists, whereas inhibition of growth signaling via the MAPK pathway might be achieved through treatment with A_{2B}AR agonists. The relative importance of these effects needs to be investigated using in vivo models before therapeutic suggestions can arise. It may eventually

turn out that both agonists and antagonists will provide useful options for treatment in combination with other therapeutic measures if used at different stages of the disease.

A_{2B}ARs KO mice exhibited significantly attenuated tumor growth and longer survival times after inoculation with Lewis lung carcinoma compared to wild-type (WT) controls. Lewis lung carcinoma tumors in A_{2B}ARs KO mice contained significantly lower levels of VEGF compared to tumors growing in WT animals. Thus, these data suggest that tumor cells promote their growth by exploiting A_{2B}AR-dependent regulation of VEGF in host immune cells [95].

It has been demonstrated that the DNA-damaging agents etoposide and doxorubicin can increase IL-8 expression in human melanoma cancer cells. In particular, it has been shown that A_{2B}AR blockade can impair IL-8 production in melanoma cells treated with etoposide and doxorubicin. This understanding may present the possibility of using A_{2B}AR antagonists to reduce chemotherapy-induced inflammatory cytokine production [96].

The pro- and anti-tumoral effects induced by A_{2B}AR activation are summarized in Fig. 3.

7. Role of A_3 adenosine receptors (A_3ARs) in cancer

A_3ARs are present in different types of tumor cells, such as HL60 and K562 human leukemia [97,98], Jurkat lymphoma [24], U937 monocytic-macrophagic human cell lines [99,100], Nb2 rat lymphoma [101], A375 human melanoma [25], PGT-beta mouse pineal gland tumor cells [102], human glioblastoma [42,103], and human prostatic cells [104]. It is well established that A_3 expression is highly sustained in tumor cells [24,25,98,102]. In more recent studies, a comparison between A_3AR expression in tumor vs adjacent and relevant normal tissues supported the assumption that the receptor is upregulated in colorectal cancer. Furthermore, there is substantial evidence showing that A_3AR expression level is directly correlated to disease severity [105]. The receptor density values of the A_3AR in colon carcinoma tissues from 73 patients revealed an increased density of this subtype in comparison to adjacent, remote, and healthy colon mucosa [105] (Fig. 4). Interestingly, large adenomas showed increased binding vs small adenomas, which had affinity and density values that were very similar to those of the mucosa of healthy subjects. An additional result of this study was that receptor affinity and density values (*K*d and *B*max, respectively) were reflected in the peripheral blood lymphocytes and neutrophils of the patients with colon carcinoma. Upon tumor resection, the A_3AR binding values returned to that of the healthy subjects, suggesting that the receptor may represent a biological marker [105]. Similar data showing higher A_3AR protein and mRNA expression levels in human melanoma, colon, breast, small-cell lung, and pancreatic carcinoma vs adjacent non-neoplastic tissue or normal tissue were reported, suggesting that the A_3AR upregulation is a phenomenon common to different malignancies [106]. Further analysis revealed that the lymphnode metastasis expressed even more A_3AR mRNA levels than the primary tumors, supporting the notion that A_3AR levels may reflect the status of tumor progression [106]. Moreover, computational analysis using different database sources supported the biological analysis that A_3AR is over-expressed in tumor tissues [106]. In a comparative study, it has been shown that primary thyroid cancer tissues express high levels of A_3ARs, as determined by immunohistochemistry analysis, whereas normal thyroid tissue samples do not express A_3ARs [107]. Accordingly, it has been reported that, except for the aorta and heart, during normal embryo development no expression of A_3ARs was found [108].

In a further study, it has been shown that A_3AR mRNA expression is upregulated in hepatocellular carcinoma (HCC) tissues in comparison to adjacent normal tissues [109]. Remarkably, upregulation of A_3AR was also noted in peripheral blood mononuclear cells (PBMCs) derived from the HCC patients compared to healthy subjects. These results further show that A_3AR in PBMCs reflects receptor status in the remote tumor tissue [109].

Taken together, the findings described above that show A_3AR over expression in different tumor cell types provide the rationale that this receptor may be utilized as a specific target to treat cancer [110].

As for the role of A_3AR in tumors, it has been reported that it is involved in the regulation of the cell cycle and both pro and anti-apoptotic effects have been reported depending on the level of receptor activation [41,99,111–114]. Starting from the observation that muscle tissues are resistant to tumor metastases, it was reported that one of the active components of muscle cell conditioned medium was adenosine, that exerted a differential effect on tumor and normal cell growth [115]. This inhibition was removed when the A_3AR was blocked, whilst it was mimicked following A_3AR agonist stimulation. The mechanism was found to involve inhibition of telomerase activity and arrest in the G0/G1 phase of the cell cycle, leading to a cytostatic effect in Nb2-11C lymphoma cells [101]. In addition it was demonstrated that A_3AR inhibited tumor growth by regulation of the WNT

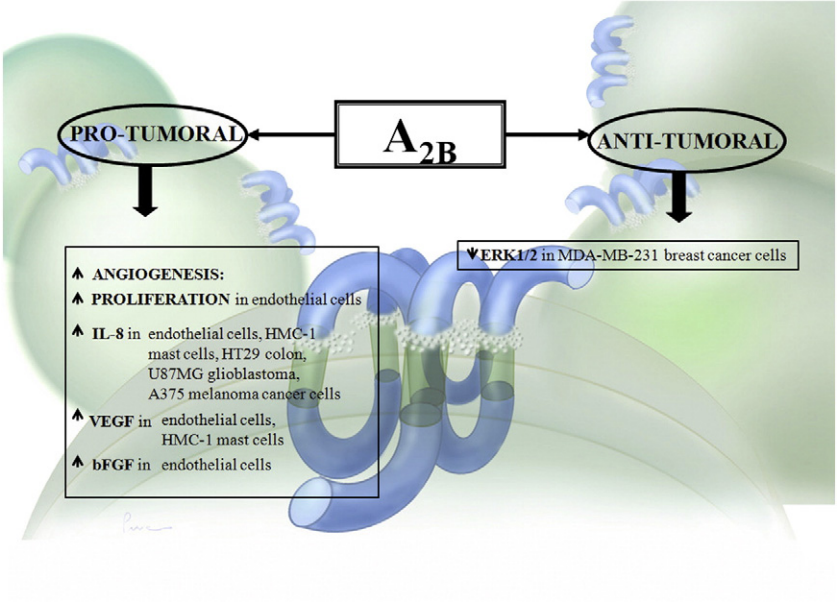

Fig. 3. Role of A_{2B}AR in cancer development. Schematic representation of the principal pathways activated by A_{2B}AR in the modulation of pro- and anti-tumoral effects.

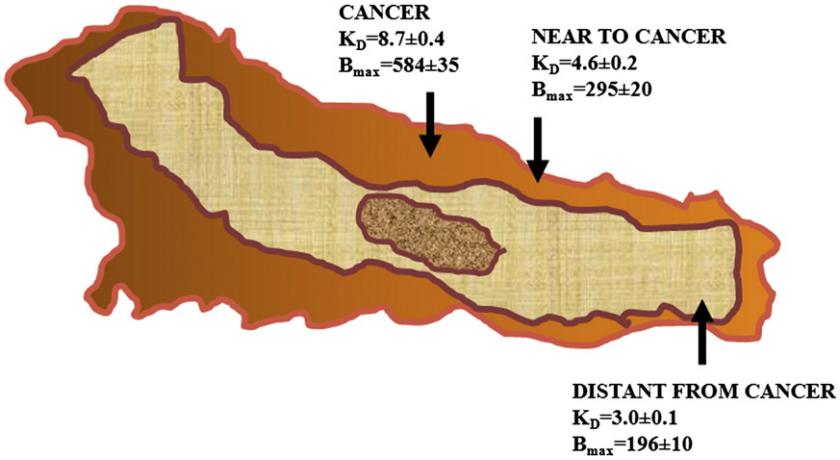

Fig. 4. The A_3AR as tumoral marker. Expression of A_3ARs in colon tumor and surrounding peritumoral normal mucosa at a distance of about 2 and 10 cm from the tumor. Kd and Bmax are affinity binding constant of [^{3}H]MRE 3008 F20 to A_3ARs and receptor density of A_3ARs, respectively, as reported in [105].

pathway [116]. The WNT pathway, active during embryogenesis and tumorigenesis, mediates cell cycle progression and cell proliferation. A key modulator of this pathway is represented by glycogen synthase kinase (GSK)-3β that is crucial for β-catenin phosphorylation. β-catenin induces the transcription of genes fundamental for cell cycle progression such as c-myc and cyclin D_1. Upon exposure of tumor cells to the A_3 agonist, a decrease in the protein expression level of A_3AR and the downstream effectors protein kinase A (PKA) and protein kinase B (PKB/Akt) was observed. Consequently, the GSK-3β protein level increased, resulting in the destabilization of β-catenin and the subsequent suppression of cyclin D_1 and c-myc expression. Methyl 1-[*N*6-(3-iodobenzyl)-adenin-9-yl]-β-Dribofuronamid (IB-MECA) treatment also induced down-modulation of the expression of NF-kB, known to regulate the transcription of cyclin D_1 and c-Myc [116,117]. IB-MECA and 2-Chloro-*N*6-3-iodobenzyladenosine-5′-*N*-methyluronamide (Cl-IB-MECA) were tested both in vitro and in vivo at low concentrations and dosages, respectively. Remarkably, at this low concentration range these agonists induced a differential effect on tumor and normal cell proliferation. Inhibition of the growth of tumor cells, including rat Nb2–11C and mouse Yac-1 lymphoma, K-562 leukemia, B16-F10 melanoma, MCA sarcoma, human LN-Cap and PC3 prostate carcinoma, MIA-PaCa pancreatic carcinoma and HCT-116 colon carcinoma, was found [101,117–123]. This effect was abolished by A_3AR antagonists [124], demonstrating that the response was A_3AR mediated. The A_3AR reduces the ability of prostate cancer cells to migrate in vitro and metastasize in vivo. In particular, it has been reported that activation of the A_3AR in prostate cancer cells reduced protein kinase A-mediated stimulation of ERK1/2, leading to lower NADPH oxidase activity and cancer cell invasiveness [104]. In a different study, the biological functions of adenosine in metalloproteinase-9 (MMP-9) regulation in U87MG human glioblastoma cells were investigated. In this case, it was revealed that A_3AR stimulation induced an increase of MMP-9 levels in U87MG cells by phosphorylation of ERK1/2, c-Jun N-terminal kinase/stress-activated protein kinase (pJNK/SAPK), PKB/Akt and finally activator protein 1 (AP-1). This effect was responsible for an increase of glioblastoma cells invasion [42].

In a series of other studies it has been found that inhibition of cell proliferation, or induction of apoptosis with the A_3 agonist, were obtained only by micromolar doses of the A_3 agonists [113,114,125,126]. In melanoma cells Cl-IB-MECA inhibited cell proliferation in an A_3AR-dependent manner [113]. IB-MECA produced cell growth inhibition in both ERα-positive cells and in ERα-negative human breast carcinoma cells. In both cell types, the introduction of an A_3AR antagonist blocked the effect of this A_3AR agonist [125,127]. Addition of the A_3AR agonist 2-chloro-*N*6-(3-iodobenzyl)-4′-thioadenosine-5′-*N*-methyluronamide (thio-Cl-IB-MECA) to HL-60 human leukemia cell cultures resulted in apoptosis [126,128]. Interestingly, an additional compound that inhibits the growth of tumor cells via A_3AR is 3′-deoxyadenosine (cordycepin), an active ingredient of *Cordyceps sinensis*, a parasitic fungus used in traditional Chinese medicine [114]. This molecule, at μM concentrations, induced a remarkable inhibitory effect on the growth of murine melanoma and of Lewis lung carcinoma tumor cells. This inhibitory effect was abolished by the A_3AR antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) [114].

Indeed, agonists to the A_3AR exert a differential effect on normal and tumor cells. In normal cells, the agonists induce the production of growth factors via induction of the NF-κB signaling pathway. In vitro studies were also confirmed by in vivo experiments, which revealed an increase in white blood cell numbers, when adenosine was administered before chemotherapy [101,117,119,120,129–131]. The molecular mechanism at the basis of granulocyte-colony stimulating factor (G-CSF) production included the upregulation of PI3K, PKB/Akt and NF-kB. In addition, it has been observed that Cl-IB-MECA potentiates the activity of NK cells in naïve and tumor bearing mice through the induction of IL-12 production. This effect was dependent on inhibition of cAMP levels and PKA expression. IL-12 is a potent stimulant of NK cells and is a cytotoxic factor that exerts a potent antitumor effect in vivo [132]. Therefore, A_3AR activation enhances NK cell activity and probably NK cell-mediated destruction of tumor cells [133].

However, the involvement of A_3AR in the inhibition of cancer development has been questioned by other authors. For example, IB-MECA and Cl-IB-MECA at μM concentrations inhibit the growth of various tumor cell lines (including NPA papillary thyroid carcinoma, HL-60 leukemia cells and U-937 lymphoma cells) in an A_3AR-independent mechanism [107,134]. This inhibitory effect was characterized by apoptosis and was not abolished by antagonism or knockdown of the A_3AR. Based on these results, it was concluded that IB-MECA or Cl-IB-MECA at high concentrations can induce tumor cell death through receptor-independent mechanisms, perhaps via active transport into the cells through the nucleoside transporters [107,134]. Furthermore, it has been previously reported that IB-MECA, at micromolar doses in breast cancer cells, inhibits cell proliferation through interaction with receptors different from the adenosine subtypes such as ER α [135]. Indeed at micromolar doses Cl-IB-MECA loses its selectivity for A_3ARs and the complicating presence of interaction with other adenosine subtypes might be involved in the final response. Furthermore, the difference between the effects induced by low and high doses of Cl-IB-MECA could be attributed to the receptor desensitization of A_3ARs [136]. Conversely, it has been demonstrated that A_3AR in retinal ganglion cells was obligatory for life [137] and it has been observed in colon cancer

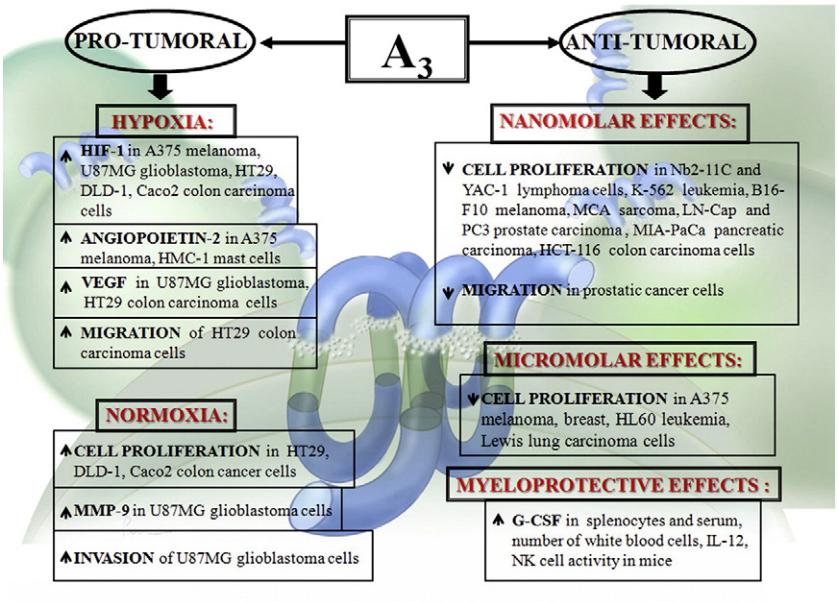

Fig. 5. Role of A_3AR in cancer development. Schematic representation of the principal pathways activated by A_3AR in the modulation of pro- and anti-tumoral effects.

cells, that after treatment with adenosine deaminase (ADA), Cl-IB-MECA increased cell proliferation through the activation of A_3 subtype and involvement of ERK 1/2 [41].

The involvement of the A_3ARs in hypoxic conditions, an in vitro model reproducing the microenvironment of solid tumors in vivo, has also been studied. Hypoxia that is typical of solid tumors [138], creates conditions that, on the one hand, are conducive to the accumulation of extracellular adenosine and, on the other hand, stabilize hypoxia-inducible factors, such as HIF-1α [12,56,139–143]. HIF-1, the most important factor involved in the cellular response to hypoxia, is upregulated across a broad range of cancer types and is involved in key aspects of tumor biology such as angiogenesis, invasion, and altered energy metabolism [144]. HIF-1 is a heterodimer composed of an inducibly-expressed HIF-1α subunit and a constitutively-expressed HIF-1β subunit [145].

The unique feature of HIF-1 is the regulation of HIF-1α expression. It increases as the cellular O_2 concentration is decreased [139,141]. During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation [141]. A growing body of evidence indicates that HIF-1 contributes to tumor progression and metastasis [146,147]. Interestingly, it has been demonstrated that A_3ARs are also over expressed in cancer tissues in comparison to normal mucosa [105]. Furthermore, attention has been paid to responses to chronic hypoxia that involve adenosine-induced changes in the transcription regulator HIF-1 expression. In particular, the correlation between AR stimulation and HIF-1α expression modulation in hypoxia has been investigated. Adenosine increases HIF-1α protein expression in response to hypoxia in human melanoma, glioblastoma and colon cancer cells [103,148,149]. These results indicate that the cell surface A_3AR transduces extracellular hypoxic signals into the cell interior. Increased HIF-1α protein synthesis through the activation of the Akt or MAPK pathways is a common theme accounting for the upregulation. To evaluate how A_3AR accumulates HIF-1α in hypoxia, the signaling pathway generated by A_3AR stimulation has been investigated and it was found that MAPK activity is required for the HIF-1α expression increase induced by A_3AR activation. Furthermore, as HIF-1α plays a key role in inducing angiogenesis, the role of adenosine in mediating the production of VEGF in tumor cells has been studied. Activation of the A_3AR in glioblastoma and colon cancer cells stimulates VEGF expression [103,149], whereas this receptor subtype promotes VEGF downregulation in pheochromocytoma cells (PC12) [150]. It has been proposed that the effect of VEGF on new capillary formation is facilitated by the concomitant stimulation of A_{2B} and A_3ARs that induce the expression of VEGF and angiopoietin-2, respectively [88]. Indeed, the activation of A_3ARs results in increased expression of angiopoietin-2 in mast and melanoma cells [88,148]. Although adenosine may contribute rather little to the increase in VEGF induced by hypoxia, it may contribute as much as 50% to angiogenesis [151]. This could mean that adenosine also acts independently of VEGF, something that is not unlikely, given the involvement of multiple cell types and multiple angiogenetic factors. Recent studies indicate that pharmacological inhibition of HIF-1α and particularly of HIF-regulated genes, that are important for cancer cell survival, may be more advantageous than therapeutic approaches based on HIF-gene inactivation. In this regard, A_3AR antagonists are able to block HIF-1α, angiopoietin-2 and VEGF protein expression accumulation in hypoxia, indicating a new approach for the treatment of cancer, based on the cooperation between hypoxic and adenosine signals. It is interesting to note that etoposide and doxorubicin affect VEGF and HIF-1 expression in human melanoma cancer cells. In particular, in blocking A_3ARs it is possible to further decrease VEGF secretion in melanoma cells, HIF-1-dependent, treated with etoposide and doxorubicin. This understanding may present the possibility of using adenosine antagonists to improve the ability of chemotherapeutic drugs to block angiogenesis [96].

The pro- and anti-tumoral effects induced by A_3AR activation are summarized in Fig. 5.

The scientific work performed in the field of cancer and ARs has led to the development of in vivo studies only for the A_3AR. Below, the principal findings obtained in animal studies will be summarized.

In all experimental models, the A_3AR agonists were administered orally due to their stability and bioavailability profile. The studies included syngeneic, xenograft, orthotopic and metastatic experimental animal models utilizing IB-MECA and Cl-IB-MECA as the therapeutic agents in melanoma, colon, prostate and hepatocellular carcinomas. Oral administration of 10–100 μg kg^{-1} IB-MECA and Cl-IB-MECA once or twice daily inhibited the growth of primary B16-F10 murine melanoma tumors in syngeneic models [124]. Moreover, in an artificial metastatic model, IB-MECA inhibited the development of B16-F10 murine melanoma lung metastases [118–120]. The specificity of the response was demonstrated by the administration of an A_3AR antagonist that reversed the effect of the agonist [124]. Furthermore, IB-MECA or Cl-IB-MECA in combination with the chemotherapeutic

Table 1
The main role of adenosine receptors in cancer.

	A_1AR	$A_{2A}AR$	$A_{2B}AR$	A_3AR
Proliferation increas in:	MDA-MB-468 breast carcinoma cells	MCF-7 breast cancer cells, endothelial cells	endothelial cells	HT29, DLD-1, Caco2 colon cancer
Proliferation decreas in:	LoVo colon, TM4 Sertoli-like, MOLT-4 leukaemic, T47D, HS578T and MFC-7 breast, glioblastoma cancer cells			Nb2-11C and YAC-1 lymphoma cells, K-562 leukemia, B16-F10 melanoma, MCA sarcoma, LN-Cap and PC3 prostate carcinoma, MIA-PaCa pancreatic carcinoma, HCT-116 colon carcinoma cells, A375 melanoma, breast, HL60 leukemia, Lewis lung carcinoma cells
Cell cycle progression in:	HeLa cervical and MDA-MB-468 breast carcinoma cells			
CDK 4 and Cyclin E expression increase in:	HeLa cervical cancer cells			
P27 inhibition in:	HeLa cervical cancer cells			
Chemotaxis increase in:	melanoma cells			
Caspases 9 and 3 increase in:	astrocytoma cancer cells	Caco-2 colon cancer cells		
Migration increase in:		endothelial cells		HT29 colon carcinoma cells
Migration decrease in:				prostatic cancer cells
VEGF increase in:		endothelial cells	endothelial cells, HMC-1 mast cells	U87MG glioblastoma, HT29 colon carcinoma cells
EPO increase in:		in Hep3B hepatoma cancer cells		
Immunoescaping increase in:		T cells		
Cell Death increase in:		A375 melanoma cells		
IL-8 increase in:			endothelial cells, HMC-1 mast cells, HT29 colon, U87MG glioblastoma, A375 melanoma cancer cells	
IL-6 increase in:			astrocytoma cells	
bFGF increase in:			endothelial cells	
ERK1/2 decrease in:			MDA-MB-231 breast cancer cells	
HIF-1 increase in:				A375 melanoma, U87MG glioblastoma, HT29, DLD-1, Caco2 colon carcinoma cells
Angiopoietin-2 increase in:				A375 melanoma, HMC-1 mast cells
MMP-9 increase in:				U87MG glioblastoma cells
Invasion increase in:				U87MG glioblastoma cells
G-CSF increase in:				splenocytes and serum, number of white blood cells, IL-12, NK cell activity in mice

agent cyclophosphamide induced an additive antitumor effect on the development of B16-F10 melanoma lung metastatic foci [119,120]. Oral administration of 10–100 μg kg^{-1} IB-MECA once or twice daily inhibited the growth of primary CT-26 colon tumors [123]. Furthermore, in xenograft models, IB-MECA inhibited the development of HCT-116 human colon carcinoma in nude mice [123]. In these studies, the combined treatment of IB-MECA and 5-fluorouracil resulted in an enhanced antitumor effect. IB-MECA was also efficacious in inhibiting liver metastases of CT-26 colon carcinoma cells inoculated in the spleen [116,120,123,152]. IB-MECA inhibited the development of PC3 human prostate carcinoma in nude mice. Additionally, IB-MECA increased the cytotoxic index of Taxol in PC3 prostate carcinoma-bearing mice

[117,120]. Finally, Cl-IB-MECA treatment dose-dependently inhibited hepatocellular tumor growth [109].

8. Conclusions

The study of adenosine and its ARs in cancer is a rapidly growing and intense area of research in drug discovery [153,154]. Adenosine, the endogenous ligand of the four AR subtypes, may activate all of these receptors in cancer due to its increased concentration in the tumor microenvironment. Its role in producing pro- and anticancer effects via each of its receptor subtypes is reported in this review (Figs. 1–3, 5). In particular, similarities and differences between receptors are easily appreciated in Table 1. It appears that all the ARs are possible targets for the development of novel approaches to the treatment of cancer. The antitumorigenic role of A_1AR in cancer was mainly studied in A_1AR KO mice, demonstrating that activation of the A_1AR on microglia inhibits the growth of glioblastomas. As for A_{2A}AR, it has been suggested that this subtype blocks antitumor immunity. In the tumor environment of hypoxia and high adenosine levels, activation of A_{2A}ARs leads to immunosuppressive effects, which decreases antitumor immunity and thus encourages tumor growth. Due to this behavior, it was suggested that the addition of A_{2A}AR antagonists to cancer immunotherapeutic protocols may enhance tumor immunotherapy. Interestingly, the safety of such compounds has already been shown in trials employing A_{2A}AR antagonists for the treatment of Parkinson's disease. The role of the A_{2B}AR in cancer is not well defined. On the one hand, activation of A_{2B}ARs leads to the release of angiogenic factors that promote tumor growth. On the other hand, the activation of A_{2B}ARs may exert an inhibitory signal on tumor cell proliferation. From these data it is not possible to understand whether agonists or antagonists will produce anticancer effects. Finally, with regard to the A_3ARs, due to their high expression in tumor cells, it has been suggested that this receptor subtype is an attractive target to combat cancer (Fig. 4). A_3AR activation with synthetic agonists induces inhibition of cell proliferation and apoptosis toward different cancer cells both in vitro and in vivo experimental models. Pre-clinical and Phase I/II studies show that these agonists are safe and well tolerated in humans and thus may be considered therapeutic agents for certain tumors such as HCC, where a significant apoptotic effect was demonstrated. However, by blocking hypoxia-induced increases of HIF-1α, angiogenesis and cell invasion in the tumor microenvironment, A_3AR antagonists may represent a successful approach for the treatment of cancer.

At present new therapeutic candidates in the fight against cancer are near to being available from the “adenosinergic system”.

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